Posttranscriptional Regulation of Cyclin B Messenger RNA Expression in the Regenerating Rat Liver

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Abstract
The growing family of cyclin genes and their products have been identified as important regulatory participants in the eukaryotic cell cycle. Cyclin proteins are currently postulated to act at the G1 restriction point, entry and exit of S phase, and the G2-M transition. We have cloned a rat cyclin B complementary DNA (cDNA) and have investigated cyclin B mRNA expression and regulation in the regenerating rat liver following 70% partial hepatectomy (PH). Sequence analysis of the rat cyclin cDNA revealed greater than 82% identity to type B1 human and murine cyclin genes. The rat cyclin cDNA was used to probe Northern blots of polyadenylated enriched RNA from regenerating rat liver from 0 through 96 h post-PH. Two species of rat cyclin B transcript were detected which mapped at 1.6 and 2.4 kilobases in length. Steady-state transcript levels began to appear around 24 h post-PH, which coincides with peak DNA synthesis. However, expression of the cyclin B transcripts peaked at 48 h and was 20-fold greater than at 24 h post-PH. Smaller peaks of expression occurred at 30 and 72 h. Run-off transcription assays using nuclei isolated at various times post-PH indicated no change in transcriptional rate during the period of regeneration. In vivo mRNA half-life determinations were performed at 24, 40, and 48 h post-PH. The half-lives of both transcript species were almost identical and determined to be greater than 12 h at 24 h post-PH, and 2.4 h at 40 and 48 h post-PH. Protein inhibition with cycloheximide increased the signal intensity of both transcripts between 48 and 54 h post-PH but had no detectable effect on 0 h transcript expression. Steady-state levels of thymidine kinase mRNA showed a similar pattern of expression by Northern analysis through 96 h post-PH as cyclin B. The present study indicates that the appearance of cyclin B mRNA in the regenerating rat liver is coincident with peak DNA synthesis, although its own peak expression is significantly delayed. Steady-state transcript levels appear to be regulated primarily by posttranscriptional events of which changes in mRNA stability may be an important determinant. We propose that the involvement of cyclin B in the cell cycle machinery is controlled at several different levels of gene expression.

Introduction
The identification of molecular components involved in cell cycle regulation, and their conservation in basic structure and function in all eukaryotes, has helped unify our understanding of the cell cycle under conditions of controlled and uncontrolled cell growth. Key to this understanding was the observation that maturation-promoting factor is composed of cyclin B and a serine/threonine kinase called p34cdc2 or CDK1 (1, 2). The maturation-promoting factor complex appears to be involved in many events during the G2-M transition of the cell cycle, including nuclear membrane breakdown, chromosome condensation, spindle assembly, and phosphorylation of numerous proteins (2–4). It is thought that in cyclin/CDK complexes, the cyclin affects the location, activity, and substrate specificity of the enzyme in a cell cycle-dependent fashion (3, 5, 6).

During the past several years, multiple cyclins and cyclin-dependent kinases have been identified from many diverse organisms (7–9). In higher eukaryotes, cyclins are classified into types based on sequence homology, expression pattern, and association with other cellular proteins (8, 10, 11). Cyclins F, G, and X are the most recently identified members of the cyclin family, although it has not been established in which phase(s) of the cell cycle each is most active (12, 13). It is currently thought that cyclins C, D, and E are involved in the G1 phase of the cell cycle, whereas cyclin A is active in the S-G2-M phase transitions. Cyclin B appears to play a critical role in the G2-M transition through its association with CDK1 (2, 14, 15).

Much work has been done to characterize the activity of the cyclin B gene product. Intracellular localization, activation of cdc25 tyrosine phosphatase, cyclin B/CDK1 biochemical targets, and degradation by the ubiquitin pathway are examples of such studies (5, 6, 16–18). However, there is a growing interest in understanding the regulation of cyclin genes, due in part to their possible involvement in onco-gensis (19). Liver regeneration provides a unique in vivo system which allows the study of cell cycle genes such as cyclin B under conditions of normal controlled cell growth (20–22). The adult hepatocyte is normally a quiescent, highly differentiated cell. However, a decrease in liver mass or traumatic cell loss induces hepatocytes to replicate. The regenerative response after 70% PH represents a fairly synchronous process in which a wave of DNA replication peaks approximately 24 h post-PH and is followed 6 to 8 h later.

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3 The abbreviations used are: CDK1, cyclin-dependent kinase 1; ASGP, asialoglycoprotein; receptor; DTT, dithiothreitol; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; MOPS, 3-(N-morpholinopropanesulfonic acid; PH, partial hepatectomy; SDS, sodium dodecyl sulfate; SSPE, sodium chloride-sodium phosphate-EDTA; cDNA, complementary DNA; bp, base pairs; kb, kilobases; poly(A)+, polyadenylated.
Cloning testes

The fragment occurs in experiments. We restored Hepatoma Box 131

We showed that cyclin B 1.2-kb fragment, Pstl/TaqI 2P-labeled, is cyclin 72 positively regulated mRNA at time of the post-PH (23), and at post-PH 1, 2, 24). Therefore, the 1.2-kb rat cyclin B clone was used to probe 0 through 96 h post-PH regenerating rat liver poly(A)⁺-enriched Northern blots. As shown in Fig. 3, cyclin B expression is first detectable by 24 h post-PH, which coincides with peak DNA synthesis of the first major synchronous wave of cell division (22, 30). Cyclin B message continues to accumulate and exhibits a small peak in steady-state mRNA levels at 30 h, and a dramatic increase at 48 h post-PH. The peak at 48 h reflects an approximately 20-fold greater transcript level than that at 24 h. The level of cyclin B transcripts drops sharply after 48 h, and a smaller peak of expression is seen 72 h post-PH. During the 96 h of regeneration, both cyclin B transcripts cycle simultaneously, although the 1.6-kb signal is 3- to 5-fold more intense than the 2.4-kb signal. No detectable cyclin B signal was observed on Northern blots containing 10 µg of poly(A)⁺-enriched RNA from sham-operated rat livers at 12, 24, and 48 h postsurgery. Thus, although cyclin B mRNA expression is detectable at the time of maximal DNA synthesis in regenerating rat liver, it peaks 24 h later and exhibits almost continuous accumulation through the first two waves of cell division in the regenerating liver.

Transcriptional Rate Analysis. It was important to determine whether transcriptional and/or posttranscriptional events were responsible for the accumulation of cyclin B steady-state transcripts in the regenerating rat liver. Run-off transcription assays were performed on nuclei isolated from livers 0 to 96 h post-PH. Surprisingly, no detectable change in transcription rate was observed above control values during the entire regenerative period. This was in contrast to the rat acute phase reactant B-fibrinogen which, used as a positive control, showed a 12-fold increase in transcription rate during the first 6 h post-PH (Fig. 4). Similar results were noted for the acute phase reactant hemopexin and the immediately protooncogenes c-fos, JunB, and c-myc. The transcriptional rate changes for each of the genes were associated with similar increases in the respective mRNA steady-state

Results

Cloning of the Rat Cyclin B Gene. A 217-bp PstI/TaqI DNA fragment of the human cyclin B1 cDNA was used to screen a rat testes cDNA library based on the results of the Northern analysis shown in the left panel of Fig. 1. This DNA fragment codes for the 5' portion of the conserved "cyclin box" region (25-27) and gave a strong 1.6-kb signal in the human hepatoma-derived HepG2 cell line as well as in rat testes. A 1.2-kb clone was positively identified for further analysis. The right panel in Fig. 1 shows the results of using the clone to probe the same Northern blot hybridized with the human box probe. Although 0 h control rat liver again revealed a strong signal, 24 h post-PH regenerating liver expressed transcripts at 1.6-kb band and, to a lesser extent, 2.4-kb. Again, testes showed only a single-sized transcript at 1.6-kb, which was now expressed more intensely than that of the human hepatoma cell line. The clone was sequenced in both directions using the double-stranded dye-deoxy chain termination method. Additional clones were isolated from the library using the entire 1.2-kb clone as the probe. The combination of the sequences from the various clones yielded a rat cyclin B 1483-bp cDNA (Fig. 2A) containing an open reading frame of 423 amino acids and a predicted molecular weight of 47,394. The rat cyclin B nucleotide sequence exhibits 82% identity with human cyclin B1 (26) and 90% identity with murine cyclin B1 (28). The complete predicted peptide sequence exhibits 87% identity with human B1 cyclin and 93% identity with murine B1 cyclin. The rat cDNA box region shows 88% identity at the nucleotide level with the human cyclin B1 box region and 93% with that of the murine cyclin B1 box region. As shown in Fig. 2B, peptide level identities between the rat cyclin B box region and those of the human and murine cyclins B1 are 94% and 96%, respectively.

Cyclin B Expression in Regenerating Rat Liver. Cyclin B protein levels in dividing cells are known to accumulate during each cell cycle with abrupt destruction at anaphase (1). It was anticipated that cyclin B transcript steady-state expression would then cycle with each wave of cell replication in the regenerating liver. In fact, it has recently been reported, using a HeLa cell cyclin B1 cDNA probe, that cyclin B1 mRNA expression is induced in regenerating rat liver (29). Therefore, the 1.2-kb rat cyclin B clone was used to probe 0 through 96 h post-PH regenerating rat liver poly(A)⁺-enriched Northern blots. As shown in Fig. 3, cyclin B expression is first detectable by 24 h post-PH, which coincides with peak DNA synthesis of the first major synchronous wave of cell division (22, 30). Cyclin B message continues to accumulate and exhibits a small peak in steady-state mRNA levels at 30 h, and a dramatic increase at 48 h post-PH. The peak at 48 h reflects an approximately 20-fold greater transcript level than that at 24 h. The level of cyclin B transcripts drops sharply after 48 h, and a smaller peak of expression is seen 72 h post-PH. During the 96 h of regeneration, both cyclin B transcripts cycle simultaneously, although the 1.6-kb signal is 3- to 5-fold more intense than the 2.4-kb signal. No detectable cyclin B signal was observed on Northern blots containing 10 µg of poly(A)⁺-enriched RNA from sham-operated rat livers at 12, 24, and 48 h postsurgery. Thus, although cyclin B mRNA expression is detectable at the time of maximal DNA synthesis in regenerating rat liver, it peaks 24 h later and exhibits almost continuous accumulation through the first two waves of cell division in the regenerating liver.

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Fig. 1. Comparison of human cyclin B1 and rat cyclin B cDNA hybridizations. Northern blots of poly(A)⁺-enriched RNA from human HepG2 cells (10 µg), rat testes (10 µg), rat 24 h post-PH liver (15 µg), and rat 0 h control liver (15 µg) are shown. Left panel, hybridized with a 32P-labeled human cyclin B1 PstI/TaqI 217-bp DNA fragment. The blot was stripped and hybridized with the 32P-labeled 1.2-kb rat cyclin B clone (right panel). Rat cyclin B transcript sizes are indicated at right. Similar results were determined in 3 separate experiments.

by mitosis (23, 24). A second, smaller peak of DNA synthesis occurs 48 h post-PH and represents a less synchronous wave of cell division. Hepatocyte as well as nonparenchymal cell replication continues until liver mass and cell number are restored.

We have cloned cyclin B from a rat testes cDNA library and have investigated its transcript expression and regulation in the regenerating rat liver. By Northern blot analysis, two cyclin B mRNA species are detectable by 24 h post-PH and peak at 48 h post-PH. Smaller peaks are exhibited at 30 and at 72 h post-PH. Our results indicate that changes in cyclin B mRNA steady-state levels in the regenerating rat liver are regulated primarily by posttranscriptional events without significant changes in transcriptional rates. This study also demonstrates the importance of mRNA stability in expression of steady-state transcripts in a well-established in vivo model of cellular proliferation.
Fig. 2. Rat cyclin B cDNA sequence and cyclin B box comparison. A, nucleotide sequence of the 1481-bp rat cyclin B clone is indicated at left, with number 1 corresponding to the translation initiation site. Amino acids for the predicted polypeptide are numbered on the right and begin with the initiating methionine (circled) as number 1. The conserved cyclin box region is underlined (25). The putative destruction box amino acid sequence (RTALGDIQN) (17) and the consensus polyadenylation site 3’ to the stop codon are also underlined. The cyclic AMP-dependent kinase consensus phosphorylation site characteristic of type B cyclins is indicated with asterisks (70). B, peptide sequence comparison of rat cyclin B, human cyclin B1, and murine cyclin B1 box regions. Amino acids are given in one-letter code. Identical amino acids are indicated by a line (1). The percentage identities are stated in the text. The amino acid residues compared for each peptide are 142 to 365 for rat B, 152 to 375 for human B1, and 149 to 372 for murine B1.
levels. Fig. 4 illustrates representative slot blots from the run-off transcription assays as well as graphic representation of the data following densitometric quantitation and normalization to the ASGPR<sub>R</sub> transcript.

**mRNA Half-Life Determinations.** Based on the invariant transcription rate of cyclin B during the regenerative period, we hypothesized that the changes in cyclin B transcript steady-state levels resulted from posttranscriptional events. Therefore, in vivo mRNA half-life determinations were made for 24, 40, and 48 h post-PH regenerating rat liver transcripts. Briefly, the transcriptional inhibitors α-amanitin and actinomycin D were injected into rats at appropriate time points post-PH. Within 30 min of injection, the combination of drugs inhibits greater than 90% of transcriptional activity as determined by nuclear run-off assays, and does so irreversibly. Poly(A)<sup>+</sup>-enriched RNA was isolated from the livers and subjected to Northern analysis. The signals obtained were densitometrically quantitated, normalized to the ASGPR<sub>R</sub>.
GP\textsubscript{c} mRNA signal, and analyzed by linear regression. In all cases, both cyclin B transcript species revealed almost identical half-lives. The apparent half-life of cyclin B mRNA at 40 and 48 h post-PH was approximately 2.4 h (Fig. 5). This is in marked contrast to the transcript half-life at 24 h post-PH which was significantly greater than 12 h. A similarly prolonged transcript half-life was determined at 18 h post-PH. No attempt was made to harvest tissue beyond 12 h postinjection. To confirm the results at 24 h post-PH, it was important to identify a short-lived mRNA half-life at that time point. Using the same mRNA samples, the p53 tumor suppressor gene transcript half-life was determined to be approximately 1.8 h.

**Effect of Cycloheximide on mRNA Expression.** The existence of cycloheximide-superinducible genes in higher eukaryotes is well established (31, 32). The effect of protein synthesis inhibition on cyclin B mRNA expression was examined in nonregenerating control liver and in regenerating liver at peak time points. Cycloheximide was injected into nonsurgerized (0 h) and 47 h post-PH rats, and livers were collected at various times up to 7 h postinjection. Control rat livers from animals given injections of vehicle alone were also harvested at 48, 50, 52, and 54 h post-PH. Poly(A)	extsuperscript{+}-enriched RNA isolated from the livers was analyzed by Northern blots. No signal increase was observed in the nonregenerating livers of cycloheximide-injected rats for periods up to 7 h. However, cycloheximide was observed to increase the signal intensity of cyclin B mRNA between 48 and 54 h post-PH as compared to vehicle-injected controls (Fig. 6). Interestingly, the increase in signal intensity was more marked for the 2.4-kb transcript than the 1.6-kb transcript. Nuclear run-off assays were performed to establish whether the increased abundance of the transcripts at 48 to 54 h post-PH was a result of transcriptional rate changes. In fact, the transcriptional activity of the cyclin B gene in the cycloheximide-treated animals was identical to that observed in the vehicle-treated animals at similar time points post-PH.

**Thymidine Kinase mRNA Expression in Regenerating Liver.** It was of interest to examine the mRNA expression of another cell cycle-participatory gene whose protein product is active beyond mid-G\textsubscript{1} phase. In fact, thymidine kinase protein is known to be active during S phase of the cell cycle. The control of its gene expression has been extensively studied in various systems and appears to involve significant posttranscriptional regulation (33-41). The steady-state expression of thymidine kinase mRNA was examined in the 0 to 96 h regenerating liver using a murine cDNA probe. As shown in Fig. 7, rat liver expresses two major transcripts, which are 2.5- and 1.2-kb in length. Both thymidine kinase mRNA species are minimally expressed at 0 h, moderately expressed about the time of peak DNA synthesis, and maxi-
Because cyclin B mRNA is undetectable in the quiescent liver, it was possible to identify its expression and regulation only during a state of cell replication. Liver regeneration after PH, particularly in rodents, has developed into a reliable in vivo system for the study of controlled cell growth and proliferation of adult differentiated cells (29, 44, 45). The fundamental elements of the system are well characterized (30). In short, it is well established that in young adult rats two major waves of DNA synthesis occur at 24 and 48 h post-PH and correspond to discrete populations of cells undergoing synchronous cell division. Cell cycle-participatory genes, such as c-fos, junB, c-myc, ras, and p53, are temporally and sequentially expressed. Moreover, liver regeneration after PH does not require chemical manipulation, nor does it involve stem cell participation. It represents a faithful, reproducible system of adult differentiated cell cycling.

In characterizing the expression of cyclin B mRNA in the regenerating liver, it was expected that the transcripts would accumulate and disappear with periodic kinetics similar to that of the cyclin B protein. It has been established in numerous cell culture and oocyte systems that cyclin B protein levels increase during each cell cycle, peak at G2-M, and abruptly degrade during mitosis (25, 26). We observed a slightly different pattern of steady-state cyclin B transcript expression in regenerating liver. A small peak in cyclin B mRNA levels occurs around 30 h, which corresponds to the G2-M phase of the first wave of cell replication. However, maximal cyclin B transcript expression is observed approximately 48 h post-PH, suggesting that, in the regenerating liver, peak steady-state transcript levels may occur during a postmitotic phase of the cell cycle. Because the second wave of DNA synthesis in the regenerating liver represents a much smaller population of cells than the first, it seems unlikely that it is responsible for the peak transcript expression observed at 48 h. The third, smaller peak in cyclin B steady-state transcript levels which occurs at 72 h may, in fact, represent the postmitotic phase of the second wave of cell replication. The lack of detectable expression in sham-operated rats at the same time points indicates that the observed cyclin B transcript levels result from a state of cell replication induced by the 70% PH.

Northern analysis of thymidine kinase mRNA in the regenerating liver provided another example of a gene whose regulation is attributed to posttranscriptional events and whose steady-state transcript levels are elevated subsequent to peak DNA synthesis. The thymidine kinase gene product is active during the DNA synthesis phase of the cell cycle. However, we have shown that, in the regenerating liver, thymidine kinase mRNA levels peak at 36 to 42 h, well beyond that of DNA synthesis, and remain elevated through 72 h post-PH. It has been shown in other cell systems that thymidine kinase mRNA and protein steady-state levels may be independently regulated (38, 39). Furthermore, thymidine kinase protein levels appear to be more tightly controlled by translational repression than its parent mRNA (37, 41). Moreover, it has also been reported that mRNA levels may decrease dramatically while the thymidine kinase gene transcription rate remains invariant (34, 40). Cyclin B gene regulation during liver regeneration appears to share certain characteristics of posttranscriptional control with thymidine kinase.

Fig. 6. Effect of cycloheximide on peak cyclin B expression in the regenerating liver. Representative Northern blots of poly(A)-enriched RNA (5 μg/lane) from 0, 48, 50, 52, and 54 h post-PH livers from rats given injections of vehicle (controls) and cycloheximide (according to “Materials and Methods”). The blot was hybridized with the 1.2-kb 32P-labeled rat cyclin B clone, stripped, and hybridized with rat c-myc to verify lane loading. Transcript sizes are indicated at right. The results are representative of 3 separate experiments.

Fig. 7. Thymidine kinase steady-state mRNA expression during rat liver regeneration. A representative Northern blot of poly(A)-enriched RNA (10 μg/lane) from 0 h control through 96 h post-PH liver shows expression of several transcript sizes (indicated at right). The blot was hybridized with 32P-labeled 1.2-kb murine thymidine kinase BamHI fragment DNA, stripped, and hybridized with the rat c-myc 32P-labeled probe to verify lane loading. The results are representative of 3 separate experiments.

Discussion

The high degree of nucleotide sequence homology to the human and murine cyclin B1 genes strongly suggests that the 1483-bp clone isolated is a rat cyclin B1 cDNA. The tissue source of the rat cyclin B cDNA and the fact that testes express only the 1.6-kb transcript imply that the full-length cDNA contains 50 to 100 nucleotides of additional 5'-untranslated region. Although murine cyclin B1 detects three transcripts in most tissues, it also detects only a single transcript in rat testes (42). Furthermore, it has been demonstrated for human cyclin A, murine cyclin B1, and several murine and human cyclin D-type genes, that the multiple transcript sizes detected by each of these cyclin genes result from differential polyadenylation sites (42, 43). Thus, the 1.6- and 2.4-kb transcripts of rat cyclin B observed in the regenerating rat liver may result from a similar type of processing.

mally expressed 36 to 42 h post-PH. Even after stringent washing of the Northern blots, the larger 4.3-kb transcript was still detectable throughout the period of regeneration. Thus, in liver regeneration post-PH, maximal expression of thymidine kinase mRNA is delayed beyond peak DNA synthesis.
cannot be explained by transcriptional rate changes. Based on this somewhat surprising observation, posttranscriptional mechanisms were addressed. The decrease in mRNA half-life observed from 24 h to 40 and 48 h post-PH provides at least a partial explanation of transcript accumulation between these time points. Unfortunately, in quiescent and early regenerating liver, cyclin B transcripts were undetectable by Northern analysis. Thus, the earliest time points available for half-life determinations were 18 to 24 h post-PH and corresponded precisely with peak DNA synthesis in the regenerating liver. Therefore, at least one mechanism by which cyclin B transcripts accumulate and cycle in the regenerating rat liver appears to involve changes in mRNA stability. Similarly, it has been reported that thymidine kinase mRNA is significantly more stable during S phase than during quiescence (33). However, this does not preclude coincident alterations in heterogeneous nuclear RNA stability and in nucleocytoplasmic processing of the cyclin B transcripts. It has been reported that the marked increase in steady-state levels of thymidine kinase mRNA in dividing BALB/c 3T3 cells results predominantly from changes in the nuclear posttranscriptional processing of its heterogeneous nuclear RNA at the G1-S boundary (35). Furthermore, the degree to which posttranscriptional processing is responsible for regulating thymidine kinase mRNA abundance during the cell cycle in CHEF/18 cells differs according to the physiological conditions of the cells (36). Thymidine kinase appears to represent just one of the cell cycle-participatory genes in which mRNA expression is not significantly regulated by transcriptional events. For example, regulation of housekeeping ribosomal protein gene expression during fetal liver development and following PH has been shown to be controlled by posttranscriptional mechanisms (46). It has also been reported that posttranscriptional events are responsible for the observed increase in multidrug resistance gene expression in the regenerating liver (47). Whereas steady-state mRNA levels demonstrated a greater than 20-fold increase for the multidrug resistance gene after PH, there was no significant increase in transcription. It was suggested that the changes in transcript levels were due to alterations in mRNA stability.

Inhibition of protein synthesis by cycloheximide resulted in increased cyclin B steady-state transcript levels in 48 to 54 h post-PH regenerating livers as compared to control PH livers at the same time points. The results are consistent with previous reports of cycloheximide-induced stabilization of protooncogenes as well as murine cyclin B1 mRNA (42, 48). Moreover, cycloheximide has been reported to extend the half-life of epidermal growth factor receptor mRNA after PH in mice (49) and also that of CCAAT enhancer-binding protein mRNA after PH in rats (50). We have determined that the effect of cycloheximide on cyclin B mRNA expression between 48 and 54 h post-PH is not due to changes in transcription rate. Also, the lack of effect of inhibiting protein synthesis on cyclin B transcript levels in nonregenerating liver suggests the existence of other nucleocytoplasmic processing events which act to control cyclin B expression in the regenerating liver. In fact, the observed changes in steady-state levels post-PH in the cycloheximide-treated animals could result from inhibition of a posttranscriptional event involving destabilization of the message, perhaps by a trans-acting labile protein (31, 51). On the other hand, by shutting down the translation machinery, cycloheximide could simply be acting to halt nonspecific translation-dependent cyclin B mRNA degradation (52).

It is well documented that the cyclin B gene product is destroyed at each cell division and must reaccumulate if mitosis is to recur (1). It is reasonable to assume that cyclin B mRNA levels must also accumulate either through transcriptional or posttranscriptional processes. However, few reports have examined the putative mechanisms by which cyclin gene expression is regulated. In contrast to prokaryotes, for which transcriptional regulation is critical, posttranscriptional control has emerged as a significant factor in the regulation of low- and medium-abundance genes in higher eukaryotes (31). For example, the regulation of human cyclin B1 mRNA expression in HeLa cells is only partially accounted for by transcriptional rate changes (26). We have shown in the regenerating rat liver that mature cyclin B transcript peak accumulation is independent of transcriptional rate changes. Rather, it appears in this unique in vivo model of cellular proliferation that the involvement of cyclin B in the cell cycle is controlled at several levels of posttranscriptional gene expression. mRNA stability appears to be at least one critical factor in the control process. However, other factors are undoubtedly involved in the regulation of cyclin B mRNA expression. They may include stabilization and processing of heterogeneous nuclear RNA and nucleocytoplasmic transport of mature mRNA. Translation-dependent factors may also participate in the regulation of cyclin B mRNA expression (53). Whatever the mechanism, there is increasing evidence to suggest that, during liver regeneration, regulation of G2-M phase genes is primarily controlled at the posttranscriptional level compared to those which are active in G1 and controlled transcriptionally. Future studies will determine whether translation of the cyclin B transcripts is involved in the regulation of their own mRNA expression.

Materials and Methods

Materials. Rats were obtained from Harlan Sprague-Dawley (Indianapolis, IN). The [α-32P]dCTP and [α-32P]UTP were purchased from Amersham (Arlington Heights, IL). Nucleotides, restriction endonucleases, calf thymus DNA, micromol guanidine isothiocyanate, and T4 DNA ligase were purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN). Agarose and RNA standards were from Bethesda Research Laboratories (Gaithersburg, MD). Sodium sarkosyl was purchased from International Biotechnologies (New Haven, CT). Long Ranger gel solution for DNA sequencing was obtained from J. T. Baker (Phillipsburg, NJ). MSI MagnaGraph membrane was obtained from Micron Separations (Westboro, MA). Oligo(dT) cellulose was from New England Biolabatories (Beverly, MA). RQ1 DNase and pGEM-3Z were purchased from Promega (Madison, WI). Cycloheximide was obtained from Sigma Chemical Co. (St. Louis, MO). RNasin, random priming labeling kit, 7-deaza-dGTP sequencing kits, urea, sucrose, HEPES, MOPS, Tris base, and phenol were from United States Biochemical Corp. (Cleveland, OH). Dulbecco’s modified Eagle’s medium and fetal calf serum were obtained from GibCO Laboratories (Grand Island, NY). All other chemicals were purchased from Aldrich Chemical Co. (Milwaukee, WI), Curtin Matheson Scientific (Eden Prairie, MN), or Fisher Scientific (Itasca, IL).

Library Screening and Cloning of the Cyclin B Gene. A rat testes oligo(dT)-primed Agt11 cDNA library (Clontech, Palo Alto, CA) was screened by cross-hybridization with [32P]-labeled human cyclin B1 (26) 1.6-kb BamHI and 217-bp
PstI/TaqI DNA fragments. Recombinant plaques were screened by standard replica plaque lift procedures. Hybridizations were done at 42°C in 5X SSPE, 35% formamide, 0.1% SDS, 5X Denhardt's, and 200 µg/ml yeast RNA. Filters were washed in 1X SSPE-0.1% SDS twice at room temperature and twice at 42°C. Isolated λ-phage inserts were verified by Southern analysis with the human cyclin B1 217-bp fragment and cloned into pGEM-3Z. Nucleotide sequences were obtained by the dideoxy chain termination method (54) using double-strand DNA template and Sequenase, Version 2.0. The sequence of the rat cyclin B cDNA was read from independent clones at least twice in both directions. Sequences were assembled using Intelligenetics software and analyzed using the University of Wisconsin Genetics Computing Group package (55).

Partial Hepatectomies and Half-Life Determinations. Sham and 70% PH were performed on male Sprague-Dawley rats (250 to 275 g) under ether anesthesia between 8 a.m. and noon (56). At various times post-PH, animals were sacrificed, and liver tissue was removed, rinsed in normal saline, and flash frozen in liquid nitrogen. Testes from control rats were obtained in a similar manner. Cycloheximide-treated animals received a 5 mg/100 g body weight i.p. injection at either 0 h or 47 h post-PH (57). Animals used for the half-life determinations of rat cyclin B mRNA received an i.v. bolus of 50 µg/100 g body weight α-amanitin. Those that were sacrificed at time points after 1 h received an i.p. injection of 150 µg of actinomycin D/100 g body weight in addition to the α-amanitin. Liver tissue was harvested at various times postinjection and processed as described above. The probe utilized in the p3 half-life determination at 24 h post-PH was a 1.5-kb EcoRI rat cDNA fragment (58).

Cell Culture. HepG2 cells (American Type Culture Collection, no. HB 8065) were maintained in Dulbecco's modified Eagle's medium containing 10% fetal calf serum as previously described (59).

RNA Preparation and Northern Blot Analyses. Total RNA was isolated from tissue and cells as previously described (60). Poly(A)⁺-enriched RNA, obtained by oligo(dT) chromatography, was electrophoresed on 1% agarose, 2.2 M formaldehyde, and 1X MOPS denaturing gels and transferred by capillary diffusion to Magnagraph nylon membranes. Probes utilized were a 217-bp PstI/TaqI fragment of the human cyclin B1 gene, a 1.2-kb EcoRI rat cyclin B insert, a 1.2-kb BamHI fragment of the murine thymidine kinase gene (61), and a 900-bp PstI fragment of the rat ASGP gene (62). Probes were labeled with [α-32P]dCTP (3000 Ci/mm0l) by random priming (63). Hybridizations of 18 to 24 h were done at high stringency (50% formamide, 42°C) for homologous probes and moderate stringency (35% formamide, 42°C) for heterologous probes. Following hybridization, membranes were washed for 15 min twice at room temperature in 1X SSPE-0.1% SDS and twice at 42°C in 1X SSPE-0.5% SDS. Autoradiography was performed using Kodak XAR film at -70°C with an intensifying screen.

Nuclear Run-off Transcription Assays. Nuclei isolated for transcription rate analyses were obtained by Dounce homogenization of tissue (1 g) in 10 ml of 0.32 M sucrose, 3 mM MgCl₂, 1 mM HEPES (pH 6.8), and 5 mM DTT. The homogenate was centrifuged for 10 min at 3,000 rpm in an SS-34 Sorval rotor at 4°C. The pellet was resuspended in 8 ml of 1.65 M sucrose, 5 mM MgCl₂, 1 mM HEPES (pH 6.8), and 5 mM DTT, layered over a 4-ml cushion of 2.1 M sucrose, 3 mM MgCl₂, 1 mM HEPES (pH 6.8), and 5 mM DTT, and centrifuged at 30,000 rpm for 1 h in an SW 40 Ti Beckman rotor. The nuclei were resuspended in 100 µl of 20% glycerol, 5 mM MgCl₂, 1 mM MnCl₂, 140 mM KCl, 25 mM Tris-HCl (pH 8.0), 1 mM DTT, 100 units RNasin, and 50 nmol each of ATP, CTP, and GTP. One hundred µCi of [α-32P]UTP (3000 Ci/mm0l) was added, and the suspension was incubated for 30 min at 30°C with periodic mixing. Nuclei were lysed by adjusting the solution to 0.3 M NaCl, and the DNA was digested by adding 12 µl of a 1 mg/ml solution of RQ1 DNase followed by a 15 min incubation at 30°C. Reactions were extracted twice with phenol:chloroform:isoamyl alcohol (25:24:1) and once with chloroform:isoamyl alcohol (24:1). The solution was adjusted to 0.3 M sodium acetate, and the RNA was precipitated twice with 3 volumes of ethyl alcohol for 30 min at -70°C. Magnagraph nylon membrane, to which 3 µg of the indicated cDNA fragment were bound according to manufacturer's instructions using a slot blot apparatus (Schleicher and Schuell, Keene, NH), were hybridized with labeled RNA at 3.0 × 10⁵ cpm/ml for 3 to 4 days under stringent conditions at 42°C. In addition to the rat cyclin B 1.2-kb and ASGP 0.9-kb cDNAs, rat hemopexin 1.0-kb and rat β-fibrinogen 1.1-kb cDNAs cloned in this laboratory, a murine c-fos 4.8-kb HindIII/BamHI fragment (64), a human junB 1.2-kb Smal/Xhol cDNA (65), a murine c-myc 2.2-kb EcoRV/HindIII fragment (66), and pGEM-3Z were used. Following hybridization, membranes were washed twice for 15 min at room temperature in 1X SSPE-0.1% SDS and twice at 42°C in 1X SSPE-0.5% SDS. Autoradiography was performed as described above.

Densitometry. Video densitometry was accomplished using a Macintosh II (Apple Computer, Cupertino, CA) coupled to a Data Translation DT2255 video digitizer (Data Translation, Marlboro, MA) and a JVC CX-N8 video camera (JVC Corp. of America, Elmwood Park, NJ) as previously described (67, 68). Quantitation of the autoradiograms utilized the NIH Image 1.4 Densitometric Analysis Program. Northern and transcriptional rate analyses were normalized with the ASGP R transcript (69).

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